Sample preparation of multiple steroids for quantitative determination by LC-MS/MS – comparison of two extraction procedures

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Introduction

Testosterone, androstenedione and 17-hydroxyprogesterone are powerful steroids which play a critical role in development and reproductive processes in men and women. The quantitative determination of serum steroids is mandatory in the investigation of suspected disorder of excessive and insufficient steroid production in adults and delayed puberty in adolescents.
While immunoassays are the general analytical method, for accurate and reproducible quantification of such analytes liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is gaining importance. Advantages are its unparalleled specificity and sensitivity combined with the very practical potential for multi-analyte quantification.

Prior to any analysis by LC-MS/MS a suitable sample clean-up is essential to efficiently remove matrix components such as proteins, lipids, carbohydrates and salt which may interfere with the analytical procedure. The Tecan AC Extraction PlateTM is an ingenious novel consumable: a 96-position, deep-well microplate featuring TICETM (Tecan Immobilized Coating Extraction) technology (Figure 1). The inner surface of each well is coated with a specific volume of sorptive material to approximately half the well's height.

The TICE coating acts as the extractant, eliminating the need for protein precipitation, liquid separation steps or the generation of emulsions. It is the basis for a simple and automatable approach to the preparation of biological samples without the need for offline operations. In parallel an automation for SLE+ plates from Biotage was set up on the same Tecan liquid handling system for comparison.

Materials and Methods

Two procedures for sample clean-up were investigated and compared:

· The **ISOLUTE SLE+ Plate** from Biotage [1] based on a supported liquid extraction procedure [2]

· The **Tecan® AC Extraction Plate™** based on TICE™ (= Tecan Immobilized Coating Extraction) technology [3]

Figure 1: The **Tecan AC Extraction Plate** (left) and the **Biotage SLE+ Plate** (right)

Calibrators, Quality Controls, Samples:
«Absolute IDQ Kit» from Biocrates (Innsbruck/Austria):
Calibrators and Quality Controls containing at total of 17 steroids including testosterone, androstenedione and 17-hydroxyprogesterone. Four calibrator levels, one blank calibrator and three control levels were used. Serum from BSB ("Blutspendedienst Bern") was used as sample. The analyte 17-Hydroxyprogesterone was spiked into the serum - the other two analytes were endogenous in sufficient concentrations.

Analysis by LC-MS/MS:
The resulting eluates / extracts were analysed using appropriate MRM mass transitions for the three steroids under investigation.

· TripleQuad5500 with DuoSpraySource
· Ionspray in positive ion mode
· Column: Phenomenex Luna 2.5 µm C18, 100 x 2 mm
· Injection volume: 40 µl

System and Workflow

Sample preparation with both plates was performed on a Freedom EVO® 150 liquid handling system equipped with 8 channels. The system was equipped with all necessary labware for both procedures (Figure 2) and sufficient space for up to 80 tubes is available on the worktable as well.

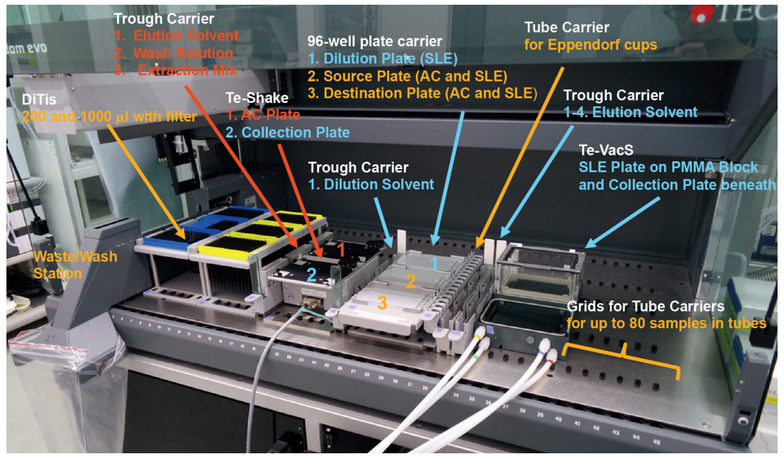

Figure 2: Configuration of the liquid handling system Freedom EVO for the automation of the two sample preparation procedures: RED: for AC Extraction Plate only · BLUE: for SLE+ Plate only · ORANGE: for both

For the **SLE+ Plate** the procedure in ref. [2] was followed. The sample was diluted and loaded on the plate by applying vacuum. Then the adsorbed steroid analytes were eluted, the eluates were dried under a flow of nitrogen and reconstituted in an appropriate solvent. The drying step was done manually off-line employing a device which distributes a flow of nitrogen into each well of the plate.

Sample preparation of a biological fluid with **AC Extraction Plate** is a simple three-step procedure:

1. Extraction step: sample (e. g. serum, plasma, urine) is mixed with buffer, shaken and removed
2. Wash step: wash solution is added, shaken and removed
3. Elution step: elution solvent is added, shaken and collected for analysis by LC-MS/MS

The last step in both procedures is the transfer of eluates into a non-coated 96-well plate ("destination plate").

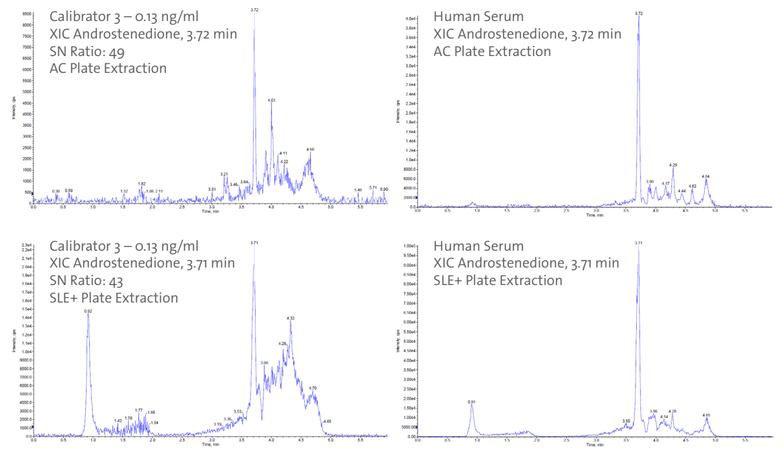

Figure 3: Extracted ion chromatograms of androstenedione extracted form Calibrator 3 and human Serum sample

Results

For both sample preparation procedures the calibration curves for the three (from a total 17) analytes were satisfactory and the accuracies of all controls where within the required limits.

Precision values for the three analytes determined in serum samples (n = 12 samples):

AC Extraction Plate:
· 2.1 – 6.8 %CV (intra-Plate, intra-Day)
· 2.6 – 11.5 %CV (intra-Plate, inter-Day)
· 1.4 – 8.2 %CV (inter-Plate, intra-Day)
· 1.7 – 8.1 %CV (inter-Plate, inter-Day)

SLE+ Plate:
· 2.3 – 10.4 %CV (intra-Plate, intra-Day)
· 2.4 – 17.3 %CV (intra-Plate, inter-Day)
· 1.0 – 25 %CV (inter-Plate, intra-Day)
· 1.2 – 25 %CV (inter-Plate, inter-Day)

Comparable LOQs were reached in both sample preparation procedures:

· Testosterone: .....0.05 ng/mL
· Adrostenedione: ..... 0.1 ng/mL
· 17-Hydroxyprogesterone: .....1 ng/mL

The determined extraction efficiencies were 70% for the SLE+ Plate and 45% for the AC Extraction Plate, respectively. The observed background noise in the mass chromatograms was distinctly lower and more reproducible in the case of the AC Extraction Plate procedure (Figure 3).

The AC Extraction Plate procedure has been automated with full walk-away capability. For a complete automation of the SLE+ Plate additionally a robotic manipulator arm will be required. Furthermore a vacuum station and an evaporator are needed which is expendable in the case of the AC Extraction Plate.

In the SLE+ Plate procedure the solvent dichloromethane is required. This solvent is highly volatile making it challenging to pipette and thus affecting overall method robustness.

Conclusion

Both sample preparation methods enable for the automation of multi-analyte samples from complex matrices for subsequent mass analysis. The AC Extraction Plate allows for a more simple protocol as just pipetting and shaking steps are required. It makes this approach thereby amenable for robotic systems with small footprints and hardware reduced to the absolute minimum: pipetting channel and on-deck shaker.

Workflow advantages of the Tecan AC Extraction Plate:

1. **Procedure requires fewer processing steps (→ more robust)**
2. **Easier to automate due to its simple "Pipette & Shake" protocol (→ lower investment)**
3. **Final eluates can directly be analyzed without the need for solvent blow-down and reconstitution (→ faster)**

References

[1] Product number 820-0200-P01 from Biotage; www.biotage.com/product-page/isolute-sle-supported -liquid-extraction-products

[2] Application Note AN740: "Extraction of Testosterone and other Steroid Hormones from Human Plasma using Isolute SLE+96-Well-Plates"

[3] Product number 30072211 from Tecan; www.tecan.com/acplate

Additional Comments

The Tecan AC Extraction Plate is for research use only – not for use in clinical diagnostics.

www.tecan.com/acplate